Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of ns-SNPs in innate immune receptors

SYNOPSIS

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biotechnology

By

RAMAN

Enrolment No. 136558

Under the supervision of

Dr. Jata Shankar

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT-173234, H.P., INDIA

AUGUST 2017

Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of ns-SNPs in innate immune receptors

Raman

Introduction

Over the last few decades, incidence of opportunistic fungal infections has increased worldwide. Among fungal pathogens, *Aspergillus* species associated infections gained importance in humans [1]. They cause infections ranging from acute to systemic in humans as well as various allergies reactions such as allergic bronchopulmonary aspergillosis (ABPA), allergic aspergillus sinusitis, hypersensitivity pneumonitis and IgE-mediated asthma [2]. Aspergillus species are one of the most ubiquitous spore bearing fungi that are present in the environment. Among all *Aspergilli*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus* cause severe infections in humans having compromised immune system as well as in immunocompetant individuals [3]. In last few years or in the last decade the number of immunocompromised patients is increased due to more number of transplantation cases, cancer, severe immunodeficiency diseases and other diseases such as HIV. These cases made human prone to opportunistic fungal pathogens [4]. *Aspergilli* associated infection in pulmonary tuberculosis and in Asthmatic patients has been alarming as reported by Denning et al. Annually at least 372,385 patients developed chronic pulmonary aspergillosis worldwide following treated of pulmonary tuberculosis [5]. Similarly, around 4,837,000 patients develop Allergic broncho-pulmonary aspergillosis out of 193 million adults with active asthma [6]. However, Study on Indian population by Agarwal et al., the ABPA burden was 1.38 million out of 27.6 million adults with asthma [7]. Apart from this, the emergence of drug resistance *Aspergilli* further complicates the aspergillus associated infections. Also, the early poor diagnosis methods of *Aspergillus* associated infections posses another threat. In last few years *Aspergillus terreus* emerged as a leading causative agent of invasive aspergillosis in severely immunocompromised patients especially in cancer patients [8]. Further, intrinsic resistance of *A. terreus* to AmphotericinB and a higher rate of invasive aspergillosis associated mortality (51% versus 30%) compared with non-terreus species of *Aspergillus* complicates the treatment regime in infected patients that lead the worrisome in medical community [9]. It appears as a frequent isolate in clinical samples in various parts of world such as M. D. Anderson Cancer Center in Houston, Texas, USA and the Medical University Hospital of Innsbruck, Austria [9]. In a recent epidemiological study, the prevalence of *A. terreus* isolates (6.6%) has been observed among *Aspergillus* infection in the duration of 4 years at referral chest hospital, Delhi, India [10]. Often *A. terreus* causes fatal disseminated infections in severe immunocompromised individuals [11]. Further, it is difficult to diagnose *Aspergillus* or fungal pathogens at an early stage due to lack of suitable diagnosis method.

The *Aspergillus* species produce thousand of conidia or 1-100 conidia per m^3 [2]. These conidia are inhaled by humans daily. In humans, lungs are the primary organ site where inhaled conidia from *Aspergilli* reside and if they are successful in germination, established invasive infection in the host [2]. Some of these inhaled conidia entrapped by upper respiratory tract and cleared. Despite of upper respiratory tract functions, they reached to lower respiratory tract due to their small size and entrapped in lungs alveoli. Thus the alveolar epithelial cells are the primary cells that encounter these inhaled conidia and hinder the establishment of invasive infection [12]. In immunocompromised persons these cells plays important role in blockade of infection due to the presence of fungal recognition receptors such as Dectin-

1, collectins and Pentraxin proteins also called as pathogen recognition receptors (PRRS) [13]. PRRs recognize these fungal pathogens by their pathogen associated molecular patterns (PAMS) and help to clear them by activation of effector molecules such as cytokines and chemokines or antimicrobial peptides [14]. If the immune system of individual is impaired or severely suppressed; conidia start to germinate and form hyphae to cause invasive or systemic infections. Thus, the transition of conidia to the germ tube followed by mycelia is a critical step in establishing systemic infection. Despite the importance of the early morphological transition, the mechanism of germination of *A. terreus* conidia is not well-understood. Necessary knowledge on mechanism on transition may expedite the development of better diagnostic and therapeutic strategy to combat invasive aspergillosis caused by *A. terreus*.

The interaction studies *in-vitro* using various immune cells and *in-vivo* studies in mice models helped to understand the mechanism of pathogenesis of *Aspergillus* species and host immune response to their infections. Furthermore the transcription studies on lung epithelial cells interacting with *A. fumigatus* suggested that in infected cells most of the up regulated genes were related to immune response, cytoskeleton rearrangement, chemotaxis and signalling pathways [15]. However, it is unclear how alveolar epithelial cells respond to *A. terreus* conidia or germinating conidia. So it is important to understand the mechanism or pathways/ proteins expressed by alveolar epithelial cells during interaction that may involve in the clearance of *A. terreus* conidia from lungs after inhalation. Proteome analysis during host-*Aspergillus* interaction may provide pivotal insight into the *Aspergillus*-mediated infections. Further proteome studies will help to establish new early diagnostic marker as well as vaccine candidates for aspergillus associated infections.

On the other side human immune system have different strategies to encounter these fungal pathogens. Immune systems of humans recognize these pathogens with the help of Pathogen recognition receptors (PRRs) such as Dectin-1 (C-type lectin) and Pentraxin-3 [16]. The recognition of pathogens leads to activate immune response to clear them from host. In last decade the single nucleotide polymorphisms (SNPs) gain importance due to their effect on the structure function of genes [17]. Thus, the SNPs in pathogen recognition receptors have deleterious effects on their structures that make individuals susceptible to pathogens [18]. The presence of Dectin-1 on lung epithelial cells and circulation of Pentraxin-3 in lungs encounter these pathogens. SNPs located in domains of these receptor proteins that recognize pathogen molecular patterns (PAMS) may alter the ability of PRRs to bind pathogens. Despite, the importance of Dectin-1 and Pentraxin-3 proteins it is still unclear how nsSNPs could affect its protective functions against human fungal pathogens, which colonize in respiratory tract and lungs. There are different efforts that have been made to reveal the effect of these nsSNPs on protein structure and functions. But still, there is a large number of SNPs data is available and to discover effects of SNPs/nsSNPs on proteins function and structure is the huge task. An effective alternative to these hurdles are various *in-silico* approaches to elucidate biochemical severity of the amino acid substitution, as well as the protein sequences and/or structural information, thus provide a more feasible approach for phenotype prediction. Keeping in view the gap existing in *A. terreus* morphogenesis, response of lung epithelial cells to *A. terreus* conidia and effect of nsSNPs in innate immune receptors, the current work on "**Proteome analysis of human lung epithelial cells interacting with *Aspergillus terreus* conidia and structure-function relation of ns-SNPs in innate immune receptors**" was undertaken with following objectives.

Objectives:

1. To elucidate the protein/enzymes or biochemical pathway of *Aspergillus terreus* conidia during the germination.
2. To study the host response during the interaction of human lung epithelial cells with *Aspergillus terreus* conidia.
3. Structure-function relation of variants in C-type lectin domain of Dectin-1 receptor to predict the susceptibility to *Aspergillus* associated infections.

Material and Methods

***Aspergillus terreus* strain and culture conditions**

Aspergillus terreus strain (NCCPF 860035) was acquired from National Culture collection of pathogenic fungi (NCCPF), PGIMER Chandigarh India. The growth of *A. terreus* (NCCPF 860035) was maintained on Sabouraud Dextrose Agar media (SDA, HiMedia, India) for the period of 7 days at 37°C to produce conidia. After that the conidia were harvested by gentle rubbing the surface of media plates using phosphate buffer saline (pH 7.4) having 0.05 % tween 20 (PBST). The conidial suspension was collected and centrifuged at 1800g for 5 min. The conidial pellet was washed with ice cold Phosphate buffer saline (PBS pH 7.4) for 4 times to remove any medium component. The purity of conidial suspension was evaluated using light microscopy. The obtained conidia were stored at 4°C for further use.

***Aspergillus terreus* morphotypes identification**

The conidia of *A. terreus* were inoculated into Dulbecco's Modified Eagle Medium (DMEM) pH 7.4 (HiMedia, India) supplemented with 10% Fetal Bovine Serum (FBS, HiMedia, India). Before inoculation the conidial viability was checked on SDA media plates. The inoculated medium was incubated at 37°C with continuous shaking. At every hour of growth, the conidial developmental changes were monitored under microscope (Magnus MPS-USB, Olympus, India). The developmental stages of *A. terreus* conidia were observed as resting conidia, swollen conidia, germinating conidia and hyphae. The more than 90% germination of *A. terreus* conidia were determined by counting 200 conidia in triplicate under light microscope.

Large scale culture of germinating conidia for protein extraction

The three flask of DMEM medium supplemented with 10% FBS were inoculated with *A. terreus* conidia and incubated at 37°C under continuous shaking (120 rpm). After achieving more than 90% germination, medium was removed by centrifuging samples at 15000 rpm for 15 minutes at 4°C. Further collected pellets of germinating conidia were washed thrice with ice-cold PBS (pH 7.4) to remove any medium components. Collected samples were snap chilled with liquid nitrogen and stored at -80°C for total protein extraction.

Culture of human lung epithelial cells for interaction study

The human lung epithelial cells A549 obtained from National cell collection centre NCCS Pune, India for the current study. Cells were maintained in DMEM medium supplemented with 10% calf fetal serum (CFS, HiMedia, India) at 37°C and 5% CO_2 under humidified conditions until confluence. The confluent A549 cells layer was treated with culture grade trypsin in phosphate buffer saline (HiMedia, India), the

obtained cell culture suspension was washed with PBS (pH 7.4) and transferred to 25 cm^2 culture flasks. Further these culture flasks were maintained under same condition as described above. After obtaining confluence of cells, these flasks were used for interaction studies.

Human lung epithelial cells interaction with *A. terreus* for proteome study

The epithelial cells A549 were grown in two culture flask at a cell density of 1x 10^6 cells, they were cultured in serum free media for 16 to 18 hours before interaction assay. After that cells were infected with viable *A. terreus* conidia in a ratio of 10:1 (multiplicity of infection between conidia and cells were, 10:1). The flasks were maintained at 37°C and in the presence of 5% CO_2 under humidified chamber. Simultaneously a control flask of A549 cells was also maintained at same condition. Further at every 2 hour flasks of interaction assay were monitored under microscope to obverse germline formation of *A. terreus*. At 16 hours interaction was stop due to germination of conidia. Interacting cells were collected along with germinating conidia by gentle scarping and further, centrifuge to remove media and washed with ice cold PBS (pH 7.4) to remove any medium residue. Simultaneously control flask of A549 cells was collected by gentle trypsinization and washed three times two remove any medium residue. The cell pellet from interacting study and control flask were snap chilled with using liquid nitrogen and stored at -80°C for protein extraction.

Total protein extraction from germinating conidia, interactome of *A. terreus* with A549 cells and control lung epithelial cells

All samples were snap chilled with liquid nitrogen and grounded with the help of mortar pestle twice to achieve fine lysate powder. Further total proteins were extracted in 50 mM Sodium phosphate lysis buffer pH 7.4 containing 2 mM EDTA, 0.2 mM DTT, and 1 mM PMSF with constant stirring for 3 hours at 4°C. After that samples were transferred to centrifuge tubes and centrifuged at 15000 rpm for 20 minutes at 4°C. The supernatant was transferred to fresh tubes and precipitate over night at -20°C using 5% trichloroacetic acid (TCA). Next day precipitated proteins were washed with chilled acetone (-20°C) four times to remove TCA. The collected protein pellets after washing were dissolved in protein rehydration buffer (2% CHAPS, 8 M urea, 25 mM DTT). Proteins concentration was determined using Bradford method and protein samples were stored at -80°C for further use.

SDS-PAGE analysis of extracted proteins from all samples (germinating conidia, interactome and lung epithelial cells alone)

Proteins of all samples from two biological replicates were separated on 12% SDS-PAGE gel [19]. The equal concentration of proteins from two biological replicates was precipitated using methanol and chloroform precipitation method. The obtained pellet after precipitation was dissolved in laemmli sample buffer contains 10% SDS, Tris–Cl (pH 6.8), 20 % glycine, 0.02 % bromophenol blue and beta-mercaptoethanol. A 12% SDS-PAGE gel was prepared using Bio-Rad protein assembly and samples were run at 70v in staking gel and 100v in separating gel. After protein separation, gel was stained with coomassie blue stain overnight and destain thereafter. The obtained gel having separated proteins was scanned on Bio-Rad G800 densitometer gel scanner.

Protein digestion

The protein samples were dissolved in 50 mM ammonium bicarbonate to obtain the required concentration of 1 mg/ ml for LC-MS/MS-QTOF analysis using Acquity Waters UPLC system (Waters).

The protein samples were analyzed at Sandor Life Sciences Pvt. Ltd, Hyderabad, India. For analysis, 100 µl of protein samples were precipitated with acetone followed by treatment with 10 mM DTT at 56°C for 1 hour. Further, samples were subjected to alkylation with 55 mM IDA at room temperature for 45 minutes in dark. Trypsin digestion was performed overnight at 37°C for protein samples. Tryptic digest was eluted in 0.1% formic acid. An aliquot of protein samples were subjected to SDS-PAGE to analyze digestion and remaining sample were subjected to the BEHC18 column for peptides separation.

LC-MS/MS and data analysis

The separated peptide fractions were analyzed using Acquity Waters UPLC system (Waters). The peptides were separated on a BEHC18 column having size 2.1mmx150mmx1.7umfor 60 minutes run using buffers (buffer A: 0.1% formic acid and buffer B: 0.1% formic acid, ACN) at a flow rate of 0.2 mL/min. The separated peptides were ionized using ESI (Electrospray Ionization) at a temperature of 275°C and spray volume of 2 kV. The mass spectra were obtained in automatic MS/MS mode. The obtained spectra were analyzed using PLGS software (Waters Inc). The data was obtained with following parameters enzyme trypsin with one missed cleavage having modification carbamidomethyl of cysteine and oxidation of methionine with peptide tolerance (ppm) 100 and fragment tolerance (ppm) 200. The data search was made against *A. terreus* in UniProt database and for interaction study the data search was made against *A. terreus* and *Homo sapiens* in UniProt Data base.

Gene ontology

The gene ontology annotations of predicted proteins were done using BLAST2go algorithms. To get the broader GO terms such as cellular components, molecular function and biological function, the GO slimmer tool was used. Further few expressed proteins involved in secondary metabolite pathways were annotated with the help of UNIPROT database.

Quantitative Real Time-PCR (qRT-PCR)

Total RNA from conidia and germinating conidia of *A. terreus* was extracted from two biological replicates using TRIzol reagent as per manufacturer's instruction (Invitrogen, USA). The quality and quantity of extracted RNA were assessed at A_{260} nm/A_{280} nm by using Nanodrop spectrophotometer (Thermo Scientific, USA). The integrity of extracted RNA was analyzed by electrophoresis using 1.2% agarose gel stained with ethidium bromide for the presence of intact ribosomal RNA bands (18S and 28S) , visualized by UV transillumination at 302 nm [20]. The cDNA synthesis was carried out from total RNA (1µg) using verso-cDNA synthesis kit (Thermo scientific, USA) according to manufacturer's instructions. cDNA synthesis was performed at 42°C for 30 min for reverse transcription followed by inactivation at 95°C for 2 min. Primers were designed for selected gene for expression study using Primer-Blast tool at NCBI. qRT-PCR was performed for selected genes (BIORAD machine CFX96, BIORAD, USA). The cDNA (1.5 µl in a total volume of 20 µl reaction) was used as template for qRT-PCR. All the reactions were performed in triplicate from two biological replicates using SYBER-Green master-mix (Thermo Scientific, USA) according to manufacturer's instructions. The thermal cycling conditions for qRT-PCR were: initial denaturation at 95°C for 3 minutes, and 39 cycle of 95°C for 10 sec, 49°C for 45 sec, 72°C for 30 sec. A multi curve was performed to check the primer specificity. Insignificant difference was observed in transcripts encoding for 40S ribosomal protein in conidia and germinating conidia, thus used as a reference gene to normalize the expression of selected genes. The "ΔΔCt" method was used to quantify the expression of selected genes in conidia and germinating conidia of *A. terreus*.

Structural variants and functional relation of deleterious nsSNPs in C-type lectin domain of Dectin-1receptor present on lung epithelial cells and Pentraxin-3 receptor circulate in lung compartments

SNP data retrieval for Dectin-1

Single nucleotide polymorphisms (SNPs) for Dectin-1 and Pentraxin-3 genes and corresponding protein sequences were retrieved from UniProt data base (UniProtKB IDQ9BXN2 and P26022). SNPs were retrieved from the NCBI dbSNP database (https:// www.ncbi.nlm.nih.gov/SNP) and the Ensembl genome browser (http://www.ensembl.org/index.html).

Prediction of functional consequences of nsSNPs

Non-synonymous SNPs (nsSNPs) were retrieved from total SNPs present in Dectin-1 and Pentraxin-3 genes and further their functional effects were analyzed by using different five *in-silico* algorithms: SIFT, polyphen version 2, nsSNP analyzer, PhD-SNP and MAPP algorithms were used for prediction of deleterious or neutral nsSNPs of Dectin-1 and pentraxin-3 genes.

Conservation profile of Dectin-1 of antigen presenting cells and Pentraxin-3 receptors

Evolutionary conservation profile of Dectin-1 and Pentraxin-3 proteins were analyzed by ConSurf web server (http://consurftest.tau.ac.il/). ConSurf estimates the evolutionary conservation of nucleic acids and amino acids in DNA/ proteins on the basis of phylogenetic relationship between homologous protein or DNA sequences. ConSurf carries out a search for close homologous sequences using BLAST or PSI-BLAST. We submitted the amino acid sequence of Dectin-1 and Pentraxin-3 receptors to ConSurf server to predict evolutionary conserved amino acid residues.

Effect on protein stability due to nsSNPs

Amino acid change in proteins affects their stability, so we predicted the change in protein stability due to change in amino acid using I-Mutant version 2. I-Mutant version 2 is a support vector machine based tool server which predicts the change in Gibbs- free energy (DDG) by subtracting the mutant protein's unfold Gibbs free energy (ΔG) from native or wild type proteins unfold Gibbs free energy (ΔG). This tool also detects the sign of increase (Inc) or decrease (Dec) in Gibbs free energy with reliability index for change in amino acid, where RI-0 indicates lowest reliability and RI-10 indicates the highest reliability. If the value of Gibbs free energy (DDG) below 0(<0) it means the stability of protein decrease, while a value above 0 (>0) indicates an increase in protein stability. During prediction of change in Gibbs free energy, the pH and temperature were set at 7 and 25°C for all nsSNP submissions.

Molecular effects of high-risk nsSNP on protein structures

The three-dimensional (3D) structural of wild-type C-type lectin domain of Dectin-1 and Pentraxin domain of Pentraxin-3 proteins were modeled using Phyre2 version-2, a web-based server. For homology modeling, the protein sequence of Dectin-1 and Pentraxin-3 proteins were retrieved from UniProt database (UniProt ID: Q9BXN2 and P26022). This tool searches multiple sequence databases mainly PDB and PFAM and generates structures based on similarity of known structure. For the generation of 3D-sturcture of C-type lectin and Pentraxin domains, FASTA sequence of the domains were submitted to Phyre2 server under intensive mode. The PDB template c2bpdB (% identity: 61, Murine Dectin-1 protein) was used by Phyre2 server for the construction of the 3D structure of C-type lectin domain of the dectin-1 protein and for Pentraxin domain, templates having PDB ID (% identity); [1sac (24%), 1b09 (24%), 4pbo (23%), and 3flp (22%)] for the modeling of 3D-structure has been used. Further, mutant structure of C-type lectin and Pentraxin domain with high-risk SNPs was generated by Swiss-PDB mutation tool by replacing wild-type amino acid with that to mutated amino acids. The structure refinement was done with the help of ModRefiner by submitting PDB files of wild-type and mutant structure of C-type lectin and

Pentraxin domain. Energy minimization was done with the help of YASARA force field minimization tool. Further modeled structures validation such as stereo-chemical properties was carried out by ProCheck.

Molecular docking of Dectin-1 with β-1, 3-glucan

The molecular docking study of C-type lectin domain of Dectin-1 protein (wild type and mutant) was carried out with AutoDock 4. The structure of β-(1-3) glucan was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The structure of β-(1-3) glucan was docked with wild-type and mutant C-type lectin domain of Dectin-1 to determine the binding energy difference between wild type and mutant structure. The Lamarckian genetic algorithm was used in AutoDock to perform the automated molecular dockings with default parameters. The number of run was set to 50 and the lowest binding energy conformation was selected for LIGPLOT analysis.

Results

Selection of germination time point of *Aspergillus terreus* conidia for proteome analysis

No significant morphological developments of conidia were observed during first 9 hours of inoculation. After that conidia expanded isotropically and swell at 10 to 11 hours of incubation in DMEM medium (Figur-1). Conidia continued to swell and polarized growth was observed at 14 hours. Approximately hours 90% conidia were observed to be germinated at 16 hours of incubation as determined by microscopic count.

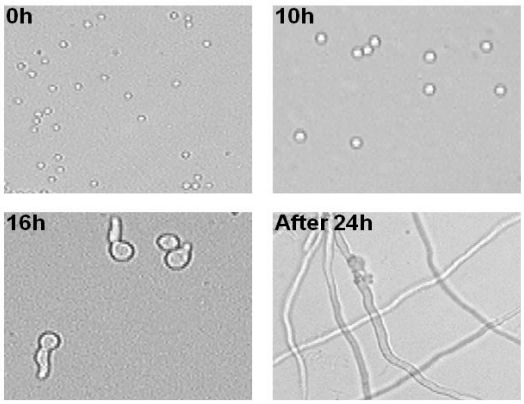

Figure-1: Morphotypes of *A. terreus* during the growth from conidia to mycelium

Identification of protein during germinating conidia

To identify the expressed protein during germination of *A. terreus* conidia (16 hours), the extracted proteins were analyzed using LC-MS/MS-QTOF. The obtained spectra were subjected to PLGS software for protein identification using data search in UniProt database. A total of 373 proteins specific to *A. terreus* were identified. Among them 74 proteins were uncharacterized. The estimated sequence coverage of identified proteins ranged from 4.2% to 80.83% and PLGS score ranged from 8.21 to 2993.38. Further the molecular weights of expressed proteins were ranged from 12949 to 228116 Da and theoretical isoelectric point (pI) ranged from 4.1 to 11. Further the gene ontology (GO) functions were assigned using BLAST2go software and UniProt database. Proteins were assigned a single GO term as biological processes, molecular function and cellular component. Biological process included protein biosynthesis

(4.01%), protein transport (6.6%), transcription/regulation (5.0%), rRNA processing (7.6%), cell wall organization (3.01%), carbohydrate metabolism/catabolism (6.02%) and other biological process showed in Figure-2. Further GO analysis revealed that the large fraction of protein expressed at germinating time point was dedicated to specific molecular functions including nucleic acid binding (14.7%), hydrolase activity (13.04%), oxidoreductase (9.3%), transferase (7.02%), ATP binding (10.36%), and other significant fraction of expressed proteins were shown in Figure-2. This analysis showed that most of the expressed proteins belonged to catalytic and binding activities. Cellular components of GO analysis showed that majority of expressed proteins were belonged to cytosol (24.41%), nucleus (20.40%), extracellular (15.05%), mitochondrion (13.71%), endoplasmic reticulum (9.36%), and rest of the significant proteins of cellular components given in Figure-2. Further general function prediction using UniProt data base revealed different functional categories of expressed proteins as "protein synthesis, amino acid metabolism and transport", carbohydrate metabolism and transport, energy production and conversion, lipid metabolism and transport, post translation modification and "secondary metabolite biosynthesis and transport.

Proteins associated with germination of *Aspergillus terreus* conidia

The analysis of expressed proteins of *A. terreus* germinating conidia permitted the recognition of a range of biochemical events and pathways associated with germination of *A. terreus* conidia.

Protein biosynthesis and amino acid metabolism

Proteins/enzymes that are involved in protein biosynthesis, amino acid metabolism, transcription, post translation modification, protein transport and DNA replication were identified during germination of *A. terreus* conidia (Figure-2). The protein synthesis requires a free pool of amino acids. We identified the proteins that involved in biosynthesis of arginin, methionine and polyaromatic amino acid biosynthesis such as ornithine carbamoyltransferase, pentafunctional AROM polypeptide, methylthioribulose-1-phosphate dehydrates, amino-acid acetyltransferase and arginine biosynthesis bifunctional protein ArgJ during germination of *A. terreus* conidia.

Proteins involved in cell wall organization and modification

Germination of fungal conidia lead to the remolding of cell wall associated proteins. The cell wall of *Aspergillus* species mainly composed of β and α-glucan and chitin. Proteins that are involved in the degradation of cell wall components have been observed during germination of *A. terreus*. Enzymes such as glucan endo-1, 3-beta-glucosidase, endopolygalacturonase, pectate lyase, alpha/beta-glucosidase and glucan endo-1, 6-beta-glucosidase have been identified. Thus, the presence of these proteins revealed the organization and modification of cell wall of *A. terreus* during the germinating.

Figure-2: Gene ontology (GO) enrichment (biological, molecular functions and cellular components) of expressed proteins in germinating conidia of *A. terreus*

Identification of probable virulence factors

Proteins involved in virulence were observed during germination of *A. terreus* conidia. Further there, homologs were mapped to *A. fumigatus* using protein BLAST. These proteins were also reviewed in literature to confirm their virulence in *A. fumigatus* knockout studies. A total 5 virulent proteins have been identified as follow cgrA (rRNA-processing protein), creB (ubiquitin carboxyl-terminal hydrolase), pepP

(Xaa-Pro aminopeptidase), myoA (myosin-1 OS), mep (metalloproteinase), hog1 (mitogen-activated protein kinase), and mpkC (mitogen-activated protein kinase).

Enzymes involved in secondary metabolite biosynthesis

Aspergillus terreus produces secondary metabolites ranging from mycotoxin (terretonin and geodin) to medically important biomolecules such as lovastatin and terrein. The enzymes involved in the biosynthesis of mycotoxins, (terretonin and geodin) have been observed. Enzymes from terretonin pathway such as methyltransferase (Trt5), FAD-binding monooxygenase (Trt3), terpene cyclase (Trt1), isomerase (Trt14), cytochrome P450 monooxygenase (Trt6), dehydrogenase (Trt9) and dioxygenase (Trt7) were identified from UniProt database. Other mycotoxin geodin pathway enzymes ACP thioesterase (GedB), anthrone oxygenase (GedH), methyltransferase (GedA), decarboxylase (GedI), atrochrysone carboxylic acid synthase (GedC), glutathione S-transferase (GedE), monooxygenase (GedF), oxidase (GedK), sulochrin halogenase (GedL) and dihydrogeodin oxidase (GedJ) have also been observed.

Quantitative expression analysis by qRT-PCR of selected genes of A. terreus in germinating conidia and conidia

In order to quantify the expression of selected proteins at transcript level at germinating conidial stage, following genes *tif35* (eukaryotic translation initiation factor 3 G), *pcy* (pyruvate carboxylase), *gedJ* (dihydrogeodin oxidase), and *ATEG_03556* (terrelysin) showed expression in conidia and in the germinating conidia. We observed significant upregulation of transcripts for terrelysin gene (885.28 folds), *pcy* (181.01 folds), *tif35* (148 folds) and *gedJ* (26.53 folds) in germinating conidia in comparison to conidia. We observed insignificant difference in transcripts encoding for 40S ribosomal protein in conidia and germinating conidia, thus used as a reference gene to normalize the expression of selected genes (Table-1).

S.No	Gene Name	Protein description	Fold difference Germinating conidia vs. conidia (16 hours vs. 0 hour)
1	*Terrelysin*	Terrelysin	885.28
2	*tif35*	Eukaryotic translation initiation factor 3 G	148
3	*pcy*	Pyruvate carboxylase	181.01
4	*gedJ*	Dihydrogeodin oxidase	26.53

Table-1: qRT-PCR analysis of transcripts of selected genes in germinating conidia in comparison to conidia of *A. terreus*

Expression of proteins in human lung epithelial cells during interaction with *A. terreus* conidia

After 16 hours of interactions with *A. terreus* conidia, the lung epithelial cells express different proteins that are involved in various cellular and biological functions during exposure to conidia.

Categories of proteins expressed in lung epithelial cells during interaction study

Gene ontology (GO) functions were assigned to expressed proteins of lung epithelia cells during their interaction with *A. terreus* conidia using BLAST2GO software and UniProt database. Proteins were assigned a single GO term as biological processes, molecular function and cellular component. Among biological processes most of the proteins were from transcription/regulation, transport, signalling, microtubule and cytoskeleton (Figure-3). Further GO analysis revealed that the large fraction of proteins specific to molecular functions including nucleic acid binding, transcription factor activity, ion binding and kinase activity. Cellular components of GO analysis showed that majority of expressed proteins were belonged to cytoplasm, nucleus, golgi apparatus, endoplasmic reticulum and cytoskeleton.

Proteins involved in cell adhesion, internalization, immune response and angiogenesis

We have observed PCDH19 and various cytoskeleton regulatory proteins such as human isoforms of phospholipase D, cadherins , cytoskeleton associated proteins and actinin proteins has been observed during interaction of lung epithelial cells with *A. terreus*. The proteins that are involved in immune response, regulation and angiogenesis were observed in interaction study. Proteins such as mannan-binding lectins, antigen processing proteins complement components, vascular endothelial growth factor receptor and various collagen proteins were observed.

Other proteins expressed by lung epithelial cells during interaction with A. terreus

Another various proteins expressed were observed such as those are involved in stress response (heat shock cognate 70 KDa), among signalling proteins serine/Threonine kinase, tyrosine kinases were also observed. The proteins that are involved in DNA/ RNA processing, transcription regulation and initiation of translation were also observed in interacting lung epithelial cells.

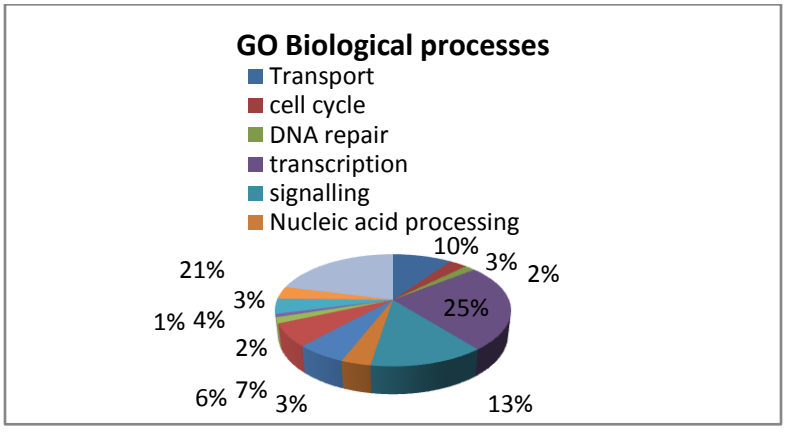

Figure-3: Gene ontology (GO) biological functions of expressed proteins in human lung epithelial cells during interaction with *A. terreus*

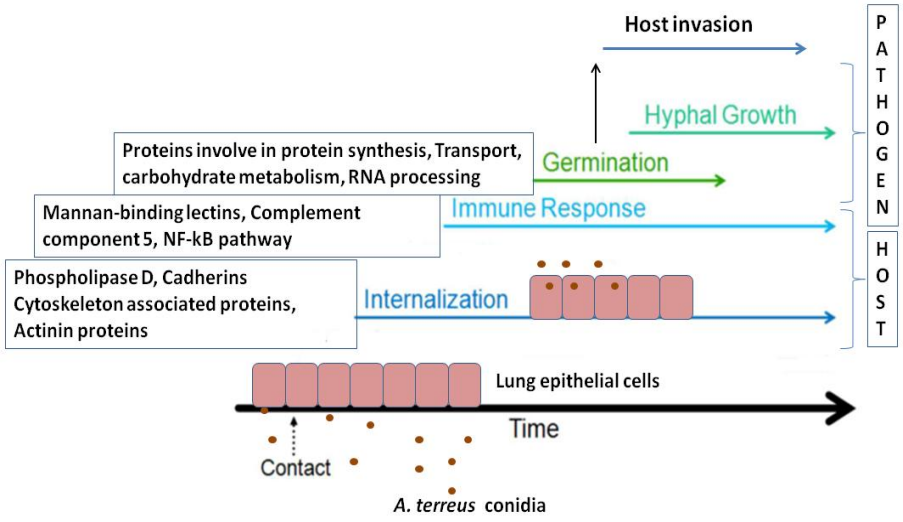

Figure-4: **Above picture has been postulated time line of events of the early interaction.** Different stages of *A. terreus* during interaction with lung epithelial cells of host are indicated on a relative time line. The events shown in the figure have been demonstrated to be occurring within the first16 to 24 of contact. As predicted with observed expressed proteins during interaction study

Major proteins/ enzymes and pathways of *A. terreus* expressed during interaction with human lung epithelial cells

During interaction over 16 hours, we observed various expressed proteins/ enzymes of *A. terreus*. We observed proteins /enzymes from secondary metabolites such as terretonin and geodin. Further the proteins/enzymes that are involved in protein biosynthesis, amino acid metabolism, transcription, post translation modification, protein transport and DNA replication were identified during interaction of *A. terreus* with human lung epithelial cells. Along with this we also observed the Terrelysin (a hemolysin) and probable putative virulence factors such as cgrA (rRNA-processing protein), creB (ubiquitin carboxyl-terminal hydrolase), pepP (Xaa-Pro aminopeptidase), myoA (myosin-1 OS), mep (metalloproteinase), hog1 (mitogen-activated protein kinase), and mpkC (mitogen-activated protein kinase) were observed. During interaction study, we have observed the expression of c-type lectin domain present in c-type lectin receptors. Thus, dectin-1 receptor having c-type lectin present of lung epithelial cells was consider to predict the structure and function relation of non-synonymous SNPs along with Pentraxin-3 receptor protein, which circulate in lung compartments.

Protein structure variation analysis in Dectin-1 (C-type lectin) and Pentraxin-3 receptor proteins
SNP Datasets
SNP datasets were evaluated for deleterious or nsSNPs in dectin-1 and Pentraxin-3 genes were retrieved from dbSNP and Ensembl genome browser. We observed 91 and 65 missense variants in dectin-1 and Pentraxin-3, which were further used to predict potential deleterious SNPs.
Identification of deleterious nsSNPs in Dectin-1 and Pentraxin-3
Various improved in-silico algorithms are available to predict potential deleterious nsSNPs from large datasets of SNPs. Five in-silico algorithms; SIFT, Polyphen-2, PhD-SNP, SNAP and MAPP were used to

analyze nsSNPs of Dectin-1 and Pentraxin-3 genes and to determine the structural and functional impact on protein structures. A total 42, 25 deleterious or 49, 40 neutral nsSNPs has been predicted by SIFT algorithm in Dectin-1 and Pentraxin-3 respectively. Only nsSNPs having SIFT score <0.05 consider as deleterious nsSNPs**.** Polyphen-2 predicted 36, 27 deleterious or damaging nsSNPs in Dectin-1 and Pentraxin-3. Further, deleterious nsSNPs were classified as highly or potential deleterious nsSNPs, when four or more than four different in-silico algorithms predicted highly deleterious nsSNPs. A total of 22 (Dectin-1) and 10 (Pentraxin-3) deleterious nsSNPs classified as highly deleterious nsSNPs. Dectin-1 protein contains a conserved extracellular domain called C-type lectin domain, which help in recognize carbohydrate moieties of fungal pathogens. C-type lectin domain contained 14 highly deleterious nsSNPs, whereas Pentraxin-3 contained 10 highly deleterious nsSNPs in Pentraxin domain that recognize fungal pathogen such as *Aspergilli* (Figure-4&5).

Figure-4

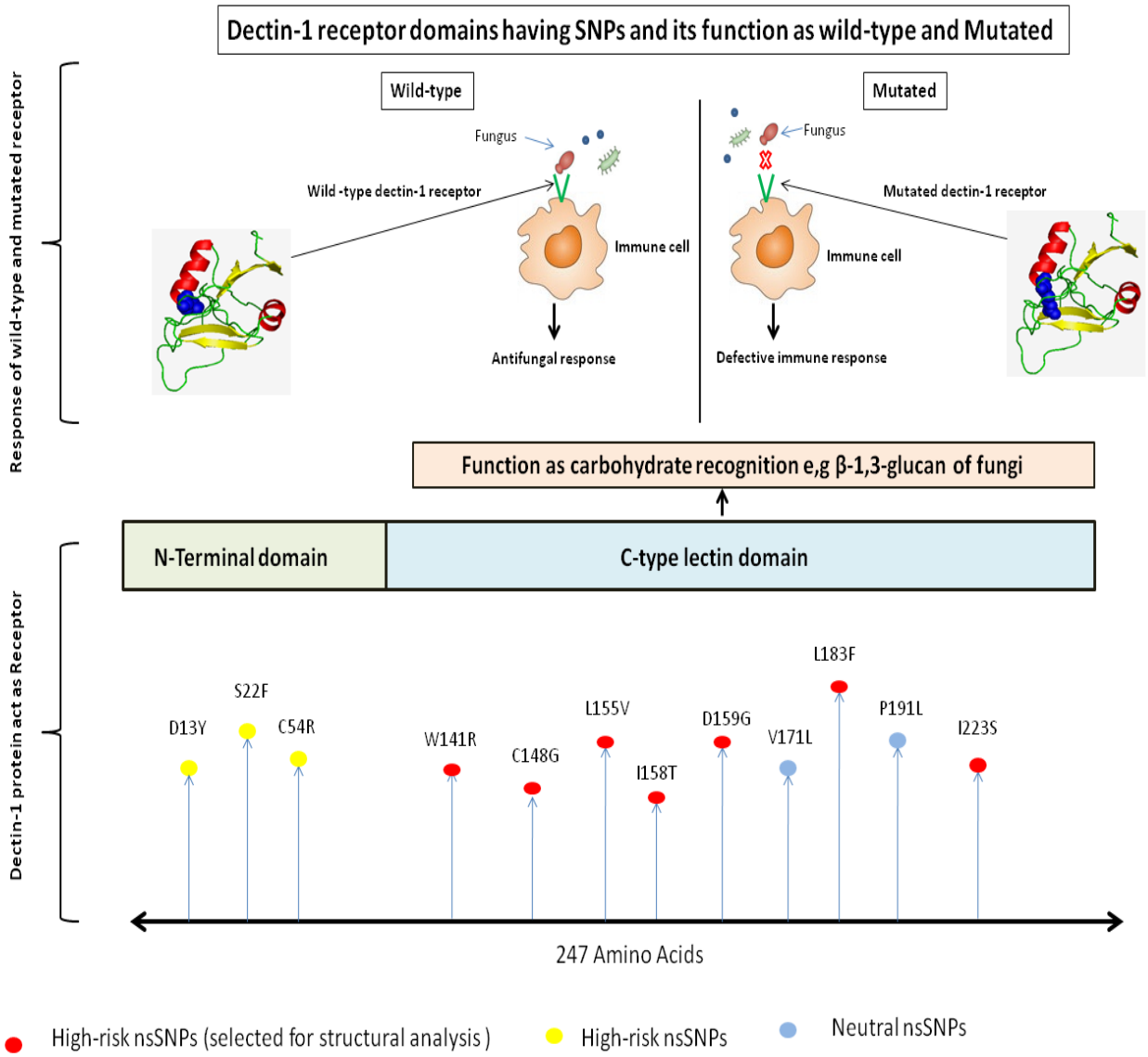

Figure-4: This figure has been generated from the results for Dectin-1. It represents the location of nsSNPs in Dectin-1 receptor and their putative effect on its function. Dectin-1 receptor protein contains 247 amino acid residues coded by six exonic region of Dectin-1 gene. C-type lectin domain recognize carbohydrate moiety of pathogens.

Figure-5

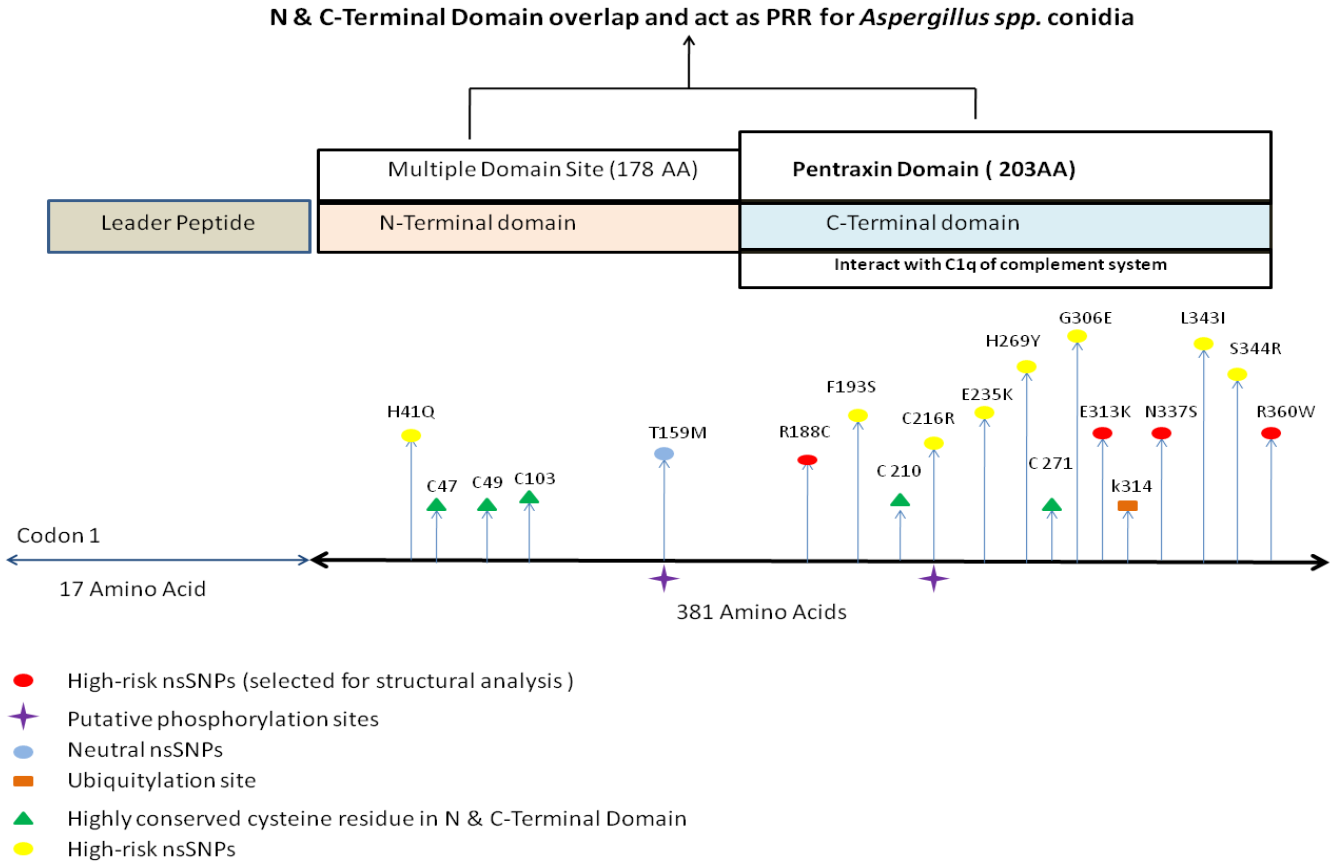

Figure-5: This figure has been generated from the results for Pentraxin-3. Figure indicates the approximate location of different high risk nsSNPs along with different post translation modification sites. It also shows the highly conserved cysteine amino acid residue in N and C- terminal domain of PTX-3 protein. PTX-3 contain N-terminal site having 178 amino acid and C- terminal site having 203 amino acid residues respectively. N-terminus contains multiple domain sites and C-terminus domain contain Pentraxin domain. Pentraxin domain interacts with C1q component of complement system to activate innate immune response. N & C- terminal domain overlap each other, interacts with various ligands and also act as PRR (pathogen recognition receptors), which recognizes *Aspergillus* conidial glactomannan [21].

Homology modeling of C-type lectin domain of Dectin-1 receptor and structure stability

Homology modeling of C-type lectin domain and Pentraxin domain of Dectin-1 and Pentraxin-3 receptors were carried out by using phyre2 web based server. Hidden Markov Method is used by Phyre2 server to generate alignments of submitted protein sequence against proteins with known 3D protein structures. Then, generated multiple alignments are used to predict homology based 3D models of the query

sequence. Phyre2 also uses an ab-initio folding simulation known as Poing to model region of a query sequence with no detectable similarities to known structures. The PDB template c2bpdB (Murine Dectin-1 protein) and 1b09 (human C-reactive protein) were used by Phyre2 server for the construction of the 3D structure of C-type lectin and Pentraxin domains of the dectin-1 and Pentraxin-3 proteins. Further, ramachandran plot analysis showed 91.7% amino acid residues in most favored region, 6.4% residues in allowed region, 1.8% residues in generously allowed region and 0% residues in disallowed region for C-type lectin domain and 98% amino acid residues in most favored region, 1.5% residues in allowed region and 0.5% residues in outlier region for Pentraxin domain. Further selected amino acid coincides in high-risk nsSNPs of C-type lectin and Pentraxin domains were used to generate variant or mutant 3D structures by substituting corresponding amino acid residues using Swiss PDB viewer. Further protein structure stability was checked using I mutant 2.0 server. Protein stability analysis was performed using I-Mutant, a neural network based tool. This tool provides scores for free energy change calculated by using FOLD-X web based tool. High risk nsSNPs in C-type lectin and Pentraxin domains were predicted to be provide less stability to protein structure as compared to wild type protein structure and free energy change ranged from -1.02 to -2.37.

Docking analysis of C-type lectin domain with β-(1-3) glucan

C-type lectin domain of Dectin-1 receptor recognizes β-(1-3) glucan a carbohydrate moiety of fungal cell wall, and thus initiates a intracellular signalling that active innate and adaptive immune response to clear fungal pathogens. The substitution of deleterious amino acid in C-type lectin domain (CTLD) may affect the binding capability of CTLD with β-(1-3) glucan. This binding affect may lead to altered immune response and thus fungal pathogen can escape mediation via Dectin-1 receptor present on innate immune cells. Hence we analyzed the binding ability of C-type lectin domain with wild type and mutant modeled structures using AutoDock 4. We have studied the interaction of C-type lectin domain with β-(1-3) glucan on the base of free binding energy, intermolecular energy RMSD and inhibition constant. The non-covalent interaction such as van der waal forces, hydrogen bonding and electrostatic forces are involved in protein and ligand interaction. Thus, we also analyzed the interaction of β-(1-3) glucan with CTLD by using LIGPLOT. Wild type C-type lectin domain showed -11.03 Kcal\mol binding energy. Lowest binding energy correlates with strong binding of ligand with their receptor. Among all mutated structure, L183F showed lowest binding energy (-12.04 Kcal\mol) where as variants I223S and L155V showed high-binding energy (-10.83 and -10.58 Kcal\mol respectively).

Discussion

Insight into germination of A. terreus conidia and probable diagnostic marker

Aspergillus terreus is an opportunistic pathogen worldwide. The germination of inhaled conidia of *A. terreus* is the key step to establish successful invasion in host tissues or organs. Thus, profiling of expressed proteins during germination of conidia not only shed light on proteins that are involved in invasion or virulence but may also provide early diagnostic markers. During germination of *A. terreus* conidia, we have identified various virulence proteins such as mitogen-activated protein kinase (mpkC), mitogen-activated protein kinase (hog1), rRNA-processing protein (cgrA) and myosin-1 that are reported as virulence factor in *A. fumigatus*. Further various proteins associated with cell wall organization have been identified, the protein involved in glucan synthesis and metabolism such as 1,3 beta-glucan synthase component FKS1, probable glucan endo-1,3-beta-glucosidase btgC, probable glucan endo-1,6-beta-

glucosidase B, mitogen-activated protein kinase hog1 and rhamno-galacturonate lyase B. Previously it has been observed that the *A. terreus* conidia and pre-swollen conidia display the β-1, 3-glucan that assist in phagocytosis by alveolar macrophages in lungs and allow persistence of *A. terreus* conidia inside phagocytic cells . Further, using qRT-PCR, it has been observed that the gene encoding for eukaryotic translation initiation factor (*tif35*) and pyruvate carboxylase (*pcy*) showed higher expression in germinating conidia in comparison to conidia. These observations suggest that during transition of conidia to germinating conidia the proteins/enzymes involved in protein synthesis and carbohydrate metabolism are required. Previously, Lamarre *et al* suggested that exit from dormancy is associated with a shift from a fermentative metabolism to a respiratory metabolism as well as a trend toward immediate protein synthesis [22].

Expression of enzymes involved in the biosynthesis of mycotoxin (Terretonin and Geodin) and other secondary metabolites (Terrein, Lovastatin and Citreoviridin) were also observed during the germination of *A. terreus* conidia. Using qRT-PCR, we have observed the high expression of *gedJ* gene, encodes enzyme dihydrogeodin oxidase of geodin biosynthetic pathway, in germinating conidia as compared to conidia**.** These observations suggest that the pathway of geodin biosynthesis may be active at germination stage of *A. terreus* but role of geodin during the germinating stage needs investigation. Recently, Ozdemir *et al* suggested the use of mycotoxin as diagnostic marker for fungal infection, but the limitation of study was they did not tested the clinical samples [23].Thus the secondary metabolites have potential for evaluation or development of noninvasive diagnostic methods for fungal infections [24].

Terrelysin, a hemolysin, was also identified during germination of *A. terreus* conidia that may contribute to virulence. Further using qRT-PCR, it has been observed the gene for encoding terrelysin showed higher expression (885.28 folds) as compared to conidia. Data suggested that synthesis of terrelysin occurs during the germination of *A. terreus* conidia. Previously it has been reported that terrelysin expressed during early growth of *A. terreus* and reduced after mycelium formation and further it diffuses extracellulary into the medium may be from apical region of hyphae or from germinating conidia [25]. Thus, identification of terrelysin protein suggests its role in invasion into the host tissues or organs. Terrelysin has been reported to lysis red blood cells and iron release, which helps fungal pathogen for their growth and metabolic activity [26]. Further, as we have observed high expression of terrelysin, it may be explored as a diagnostic marker for the diagnosis of *A. terreus* associated infections. Further human lung epithelial cells interaction study shed light on response of first line of defense cells during interaction with *A. terreus* conidia

Insight into the interaction of lung epithelial cells with Aspergillus terreus conidia

Aspergillus terreus employs unique strategy to evade human immune system. The prolonged persistence inside immune cells such as macrophage helps pathogens to escape from immune system or immune cell surveillance [11]. When the individual's immunity is compromised, conidia start to germinate and establish fatal invasive pulmonary or secondary organ infections. Invasion of host tissues need virulence factors and among *Aspergillus* species, several virulence factors has been reported in *A. fumigatus*. Aspergillus spp. conidia are the infectious agents that by pass the mucocilliary clearance and penetrate lung alveoli due to their small size. The interaction of conidia with lung environment is important, in most of the healthy persons the interaction results in clearance whereas in susceptible individuals infection

initiates. In the lungs epithelial cells are the first who encounter conidia. The interaction between epithelial cells and conidia is multifaceted and complex and has implication for later steps in pathogenesis. Increasing evidences have demonstrated a key role for the lung epithelium in response to respiratory pathogens, particularly at early stages of infection; therefore elucidating the early stages of interaction of conidia with lung epithelial cells is essential to understand the establishment of infection in cohorts of at risk patients. The interaction of conidia with lung epithelia cells initiates with adhesion and internalization of conidia. In interaction study we observed the expression of human isoforms phospholipase D, cadherins, actinin; activity regulated cytoskeleton-associated protein and C-type lectin domain proteins. It has been previously reported that phospholipase D participate in internalization of conidia and its activity directly linked with Dectin-1 receptor. This receptor contains c-type lectin doamin, which recognaise fungal pathogens[27]. Further it has been observed that cadherins proteins involved in phagocytosis of conidia by alveolar epithelial cells (A549) [28]. Furthermore the expression of genes encoding proteins actinin and activity regulated cytoskeleton-associated protein demonstrated that they involved in cytoskeleton rearrangement and thus involved in internalization of *A. terreus* conidia [15]. The contact of conidia with alveolar epithelial cells initiates various signalling pathways that activate various immune responses to conidia and germinating conidia. We observed various regulatory proteins involved in NF-$_k$B pathway. This suggested that this pathway may active during interaction with conidia or germinating conidia and activated the production of chemokines and cytokines. Previously the secretion of interleukin-8 and CXCL8 has been observed by alveolar epithelial cells during interaction with *A. fumigatus* under the control of NF-$_k$B pathway [29]. Thus the activation of these chemokines involved in trafficking of neutrophils toward infected area or in lungs and further help to clear conidia.

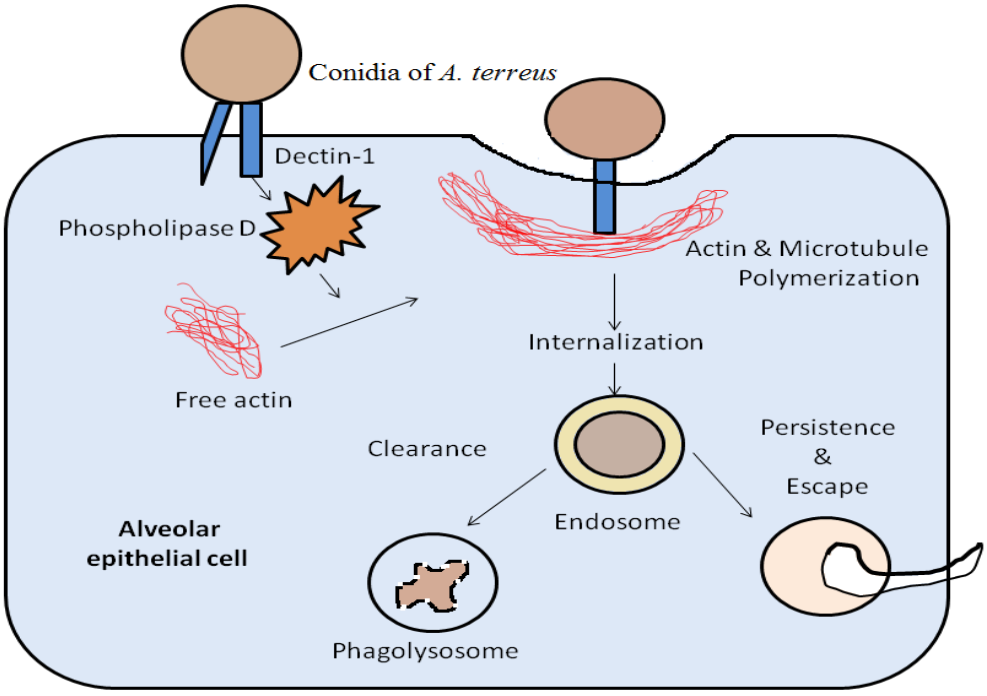

Figure-6: **Model of internalization of *A. terreus* conidia by alveolar epithelial cells has been hypothesized from the current results and previous studies on *Aspergilli*.** Once conidia swell, β-glucan in the conidial cell wall is recognized by dectin-1, activating phospholipase D and promoting actin polymerization. Conidia are then internalized through actin and microtubule polymerization and

phagocytosis. Upon internalization, they are trafficked through the endosomal system where they are degraded and cleared from the host or alternatively, small numbers may persist, germinate and escape.

Thus overall expression profile of alveolar epithelial cell interacting with *A. terreus* suggests that the internalization of conidia required microtubules and actin polymerization which indicates a mechanism similar to phagocytosis. But previously it has been observed that the cultured epithelial cells only intake up to 30% conidia and further some of them escape or persist inside cells and thus lead to germination and invasion of host tissues. During interaction study, we have observed the expression of c-type lectin domain present in c-type lectin receptors. Thus, dectin-1 receptor having c-type lectin present of lung epithelial cells was consider to predict the structure and function relation of non-synonymous SNPs along with Pentraxin-3 receptor protein, which circulate in lung compartments.

Impact of nsSNPs on innate immune receptors

In humans, lungs are the primary organ site where inhaled conidia from *Aspergillus* species reside and if it is successful in germination, establish invasive infection in the host. The bronchial and alveolar epithelial cells are the primary cells that encounter these inhaled conidia and hinder the establishment of invasive infection. In immunocompromised persons these cells play important role in blockade of infection due to the presence of fungal recognition receptors (PRRs) such as Dectin-1(C-type lectin) and Pentraxin-3 proteins. These receptors recognize fungal pathogens by their pathogen associated molecular patterns (PAMS) and help to clear them by activation of effector molecules such as cytokines and chemokines or antimicrobial peptides. In last decade the single nucleotide polymorphism (SNPs) gain importance due to their effect on the structure function of genes. Thus the understanding of nsSNPs not only provides the insight into disease susceptibility but also highlight the drug targets and biomarkers. Further, alternation in proteins structure and function can account an individual susceptible to diseases.

We utilized five in-silico algorithms to predict deleterious and neutral nsSNPs in Dectin-1 receptor and Pentraxin-3 protein, specifically SIFT, Polyphen2, PhD-SNP, SNAP and MAPP. SIFT predicted 42(46%) & 25(38%) deleterious and 49 (54%) & 40(62%) neutral nsSNP, PhD-SNP identified 31(34%) & 18(28%) deleterious and 60 (66%)& 47(72%) neutral nsSNPs, PolyPhen predicted 36(40%) & 27(41%) deleterious and 55(60%) & 38 (59%) neutral nsSNPs, where as SNAP and MAPP determined 20(22%), 54(59%) & 29(45%), 37(57%) deleterious and 71(78%), 37(41%) & 36(55%), 28(43%) neutral nsSNPs for Dectin-1 and Pentraxin-3 respectively. the difference in prediction by these tools might be the algorithms or features used by these tools. Previously various studies used these in-silico tools for prediction of deleterious and neutral nsSNPs.

By comparing all five in-silico algorithms and conserve domain analysis, 7 nsSNPS (W141R, C148G, L155V, I158T, D159G, L183F, and I223S) were observed to be high-risk nsSNPs that were located in C-type lectin domain of Dectin-1 and 4 nsSNPs (E313K, R188C, N337S and R360W) were present in Pentraxin domain of Pentraxin-3 protein. Further these high-risk nsSNPs were subjected to structure, function analysis. The murine Dectin-1 protein c2bpdB (% identity: 61) and 1b09 (human C-reactive protein) were used as template by Phyre2 software to construct 3D structure of C-type lectin and Pentraxin domains. Previously Yadav *et al* also used murine Dectin-1 protein structure to model 3D protein structure of *Bulbalus bulbalis* with 58.91 % sequence similarity [30]. I-mutant analysis showed that, there is change in protein stability or protein stability decreased after substituting amino acid in wild type Dectin-1 receptor protein. The high change in energy or stability of protein (-2.37) was observed in high-risk nsSNP (rs16910527) by substitution of isoleucine by serine at 223 amino acid position followed

by I158T (-2.15) and D159G (-1.50) in Dectin-1 receptor. Thus the overall analysis showed that these high risk nsSNPs may affect the structure and function of Dectin-1 receptor. Previously wolf *et al* showed that the two SNPs in coding region of Dectin-1 receptor lead to loss of phagocytic function. They demonstrate that the amino acid 223 play an essential structural role in Dectin-1 receptor and substitution of this amino acid may affect receptor's function [31]. Further various studies showed that the presence of Y238X polymorphism in Dectin-1 receptor impaired its function and has been associated with invasive infection of aspergillosis in high-risk patients such as those undergo bone marrow transplant or having impaired immunity [32,33]. In-vitro and in-vivo studies on Y238X polymorphism suggested that it impaired production of cytokines such as interferon-γ and interleukine-10. Thus modulate the immune response of susceptible individuals to fungal pathogens. Further, Dectin-1 interaction with other proteins was annotated by using STRING database. This database predicts the binding interaction of Dectin-1 receptor with TLR-2, CD37 and other proteins. Ferwerda *et al* showed that TLR-2 and Dectin-1 receptors work synergistically in response to fungal infection and initiate cross talk signalling and cytokines productions [34]. Recently, Fischer *et al* showed that Dectin-1 and TLR-2 polymorphisms are the predisposing factors for invasive fungal infections in acute myeloid leukemia patients [38]. Further Dectin-1 also interacts with CD37 leukocyte-specific tetraspanin that co-localized with Dectin-1 on antigen presenting cells. It has been suggested that their interaction is important for Dectin-1 stabilization in antigen presenting cell membrane and cytokine production [35]. Thus, the high-risk nsSNPs present in Dectin-1 receptor or in C-type lectin domain may affect the interaction between TLR-2, CD37 and other proteins and may abolish the downstream signalling that could affect cytokine production and immune response against fungal pathogens. Furthermore I-mutant analysis of nsSNPs in Pentraxin domain suggested that high risk nsSNPs N337S and R360W showed less stability as compared to wild type proteins, with free energy change values of -0.81 and -0.34 respectively. Recent studies showed that nsSNP A48D (rs3816527) in PTX-3 gene is associated with invasive aspergillosis in patients that undergo hematopoietic stem cell transplantation.

Dectin-1 receptor recognizes fungal pathogens through β-(1-3) glucan, a carbohydrate moiety exposed to extracellular environment during cell wall modification [41]. Thus docking study has been performed to analyze the effect mutations on binding capacity of C-type lectin domain of Dectin-1 receptor. The high-risk mutations (W141R, C148G, L155V, I158T, D159G, and I223S) observed to be unfavorable for β 1, 3 glucan binding with CTLD of Dectin-1 receptor. The lowest binding energies (-11.03 Kcal\mol and -12.04 Kcal\mol) were observed in native or wild type or a mutant (L183F) C-type lectin domain of Dectin-1 receptor. The lowest binding energies of these complexes suggested their good interaction and compatibility with β-(1-3) glucan. The higher binding energies were observed in I223S and L155V variants (-10.83 Kcal\mol and -10.58 Kcal\mol) respectively, which indicates their defective binding with C-type lectin domain of Dectin-1 receptor. Previously it has been explored that the nsSNPs influence the binding of ligand molecules with their receptors [36]. Further, to gain insight into ligplot analysis was done, which suggested that the change in hydrogen bond distance with interacting amino acid residues and ligand molecules, could affect the thermodynamic stability of ligand-receptor complex. Thus it may be possible that these high risk nsSNPs may impair the function of Dectin-1 and Pentraxin-3 receptors as pathogen recognition receptor and further affect their phagocytosis and effector function of phagocytic cells.

Conclusion and future prospects

Current work has provide insight into the germination of *A. terreus* conidia, response of lung epithelial cells to *A. terreus* conidia and affect of nsSNPs on innate immune receptors that recognize *Aspergillus* species conidia, germinating conidia and hyphae, thus help them to clear from human host. The results from germination of *A. terreus* conidia suggested that during transition of conidia to germinating conidia, the proteins/enzymes involved in protein synthesis and carbohydrate metabolism are required, and the expression of proteins terrelysin and enzymes involved in biosynthesis of secondary metabolite could pave way for development of metabolite based diagnostic marker for *A. terreus* associated infections. Further, future interaction studies to elucidate the function of secondary metabolites of *Aspergilli* interacting with respiratory epithelium will help to understand dynamic interplay between host and pathogens. An improved understanding of these interactions could lead to more effective treatment outcomes and new diagnostic markers. Further more studies on affect of nsSNPs on innate immune receptors showed deleterious effect on structure and subsequently to the function of Dectin-1(c-type lectin) and Pentraxin-3 receptors. These receptors previously known to interact with other proteins and receptors such as complement components of immune system and toll like receptor-2. Their interaction with other proteins and receptors initiate downstream function to clear fungal pathogens from infected host. Due to presence of high risk nsSNPs, it could be possible that the domain of these proteins may not be functionally active and humans with these nsSNPs in their genomes may susceptible to infection for selected pathogens. For future prospective, to establish new early diagnostic markers for invasive aspergillosis, it is necessary to carry out the in-vitro studies using mice model of invasive aspergillosis and clinical studies (patients having invasive aspergillosis) for detection of secondary metabolites or study of their kinetics in infected patient sera. These studies will help to establish new early diagnostic markers for *Aspergillus* species associated infections. Furthermore future population based studies on high risk nsSNPs will provide the early biomarker or individuals susceptibility to fungal infections.

Refrences

[1] Seyedmousavi S, Guillot J, Arné P, de Hoog GS, Mouton JW, Melchers WJG, Verweij PE (2015) Aspergillus and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease. Medical mycology 53 (8):765-797. doi:10.1093/mmy/myv067

[2] Latge JP (1999) Aspergillus fumigatus and aspergillosis. Clinical microbiology reviews 12 (2):310-350

[3] Sugui JA, Kwon-Chung KJ, Juvvadi PR, Latgé J-P, Steinbach WJ (2014) Aspergillus fumigatus and Related Species. Cold Spring Harbor Perspectives in Medicine. doi:10.1101/cshperspect.a019786

[4] Gow NAR, Netea MG (2016) Medical mycology and fungal immunology: new research perspectives addressing a major world health challenge. Philosophical Transactions of the Royal Society B: Biological Sciences 371 (1709):20150462. doi:10.1098/rstb.2015.0462

[5] Denning DW (2011) Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. BullWorld HealthOrgan 89:864–872. doi:doi:10.2471/blt.11.089441

[6] Denning DW, Pleuvry A, Cole DC (2013) Global burden of chronic pulmonary aspergillosis complicating sarcoidosis. European Respiratory Journal 41 (3):621-626. doi:10.1183/09031936.00226911

[7] Agarwal R, Denning DW, Chakrabarti A (2014) Estimation of the burden of chronic and allergic pulmonary aspergillosis in India. PloS one 9 (12):e114745. doi:10.1371/journal.pone.0114745

[8] Hachem R, Gomes MZ, El Helou G, El Zakhem A, Kassis C, Ramos E, Jiang Y, Chaftari AM, Raad, II (2014) Invasive aspergillosis caused by Aspergillus terreus: an emerging opportunistic infection with poor outcome independent of azole therapy. The Journal of antimicrobial chemotherapy 69 (11):3148-3155. doi:10.1093/jac/dku241

[9] Blum G, Hortnagl C, Jukic E, Erbeznik T, Pumpel T, Dietrich H, Nagl M, Speth C, Rambach G, Lass-Florl C (2013) New insight into amphotericin B resistance in Aspergillus terreus. Antimicrobial agents and chemotherapy 57 (4):1583-1588. doi:10.1128/aac.01283-12

[10] Kathuria S, Sharma C, Singh PK, Agarwal P, Agarwal K, Hagen F, Meis JF, Chowdhary A (2015) Molecular epidemiology and in-vitro antifungal susceptibility of Aspergillus terreus species complex isolates in Delhi, India: evidence of genetic diversity by amplified fragment length polymorphism and microsatellite typing. PloS one 10 (3):e0118997. doi:10.1371/journal.pone.0118997

[11] Slesiona S, Gressler M, Mihlan M, Zaehle C, Schaller M, Barz D, Hube B, Jacobsen ID, Brock M (2012) Persistence versus escape: Aspergillus terreus and Aspergillus fumigatus employ different strategies during interactions with macrophages. PloS one 7 (2):e31223. doi:10.1371/journal.pone.0031223

[12] Margalit A, Kavanagh K (2015) The innate immune response to Aspergillus fumigatus at the alveolar surface. FEMS Microbiology Reviews 39 (5):670-687. doi:10.1093/femsre/fuv018

[13] Inoue M, Shinohara ML (2014) Clustering of Pattern Recognition Receptors for Fungal Detection. PLoS Pathogens 10 (2):e1003873. doi:10.1371/journal.ppat.1003873

[14] Swidergall M, Filler SG (2017) Oropharyngeal Candidiasis: Fungal Invasion and Epithelial Cell Responses. PLoS Pathogens 13 (1):e1006056. doi:10.1371/journal.ppat.1006056

[15] Chen F, Zhang C, Jia X, Wang S, Wang J, Chen Y, Zhao J, Tian S, Han X, Han L (2015) Transcriptome Profiles of Human Lung Epithelial Cells A549 Interacting with Aspergillus fumigatus by RNA-Seq. PloS one 10 (8):e0135720. doi:10.1371/journal.pone.0135720

[16] Thakur R, Anand R, Tiwari S, Singh AP, Tiwary BN, Shankar J (2015) Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells? Frontiers in microbiology 6:429. doi:10.3389/fmicb.2015.00429

[17] Dabhi B, Mistry KN (2014) In silico analysis of single nucleotide polymorphism (SNP) in human TNF-alpha gene. Meta gene 2:586-595. doi:10.1016/j.mgene.2014.07.005

[18] George Priya Doss C, Nagasundaram N, Chakraborty C, Chen L, Zhu H (2013) Extrapolating the effect of deleterious nsSNPs in the binding adaptability of flavopiridol with CDK7 protein: a molecular dynamics approach. Human genomics 7:10. doi:10.1186/1479-7364-7-10

[19] Tiwari S, Thakur R, Goel G, Shankar J (2016) Nano-LC-Q-TOF Analysis of Proteome Revealed Germination of Aspergillus flavus Conidia is Accompanied by MAPK Signalling and Cell Wall Modulation. 181 (11-12):769-786

[20] Shankar J, Wu TD, Clemons KV, Monteiro JP, Mirels LF, Stevens DA (2011) Influence of 17β-Estradiol on Gene Expression of Paracoccidioides during Mycelia-to-Yeast Transition. PloS one 6 (12):e28402. doi:10.1371/journal.pone.0028402

[21] Thakur R, Shankar J (2016) In silico Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens. Frontiers in microbiology 7:192. doi:10.3389/fmicb.2016.00192

[22] Lamarre C, Sokol S, Debeaupuis JP, Henry C, Lacroix C, Glaser P, Coppee JY, Francois JM, Latge JP (2008) Transcriptomic analysis of the exit from dormancy of Aspergillus fumigatus conidia. BMC genomics 9:417. doi:10.1186/1471-2164-9-417

[23] Ozdemir HG, Kandemir H, Curuk A, Ilkit M, Seyedmousavi S (2016) Infrequent Production of Xanthomegnin by Fungal Strains Recovered from Patients with Ocular Mycoses. Mycopathologia 181 (3-4):241-246. doi:10.1007/s11046-015-9970-6

[24] Koo S, Thomas HR, Daniels SD, Lynch RC, Fortier SM, Shea MM, Rearden P, Comolli JC, Baden LR, Marty FM (2014) A breath fungal secondary metabolite signature to diagnose invasive aspergillosis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 59 (12):1733-1740. doi:10.1093/cid/ciu725

[25] Nayak AP, Blachere FM, Hettick JM, Lukomski S, Schmechel D, Beezhold DH (2011) Characterization of recombinant terrelysin, a hemolysin of Aspergillus terreus. Mycopathologia 171 (1):23-34. doi:10.1007/s11046-010-9343-0

[26] Nayak AP, Green BJ, Beezhold DH (2013) Fungal hemolysins. Medical mycology 51 (1):1-16. doi:10.3109/13693786.2012.698025

[27] Han X, Yu R, Zhen D, Tao S, Schmidt M, Han L (2011) β-1,3-Glucan-Induced Host Phospholipase D Activation Is Involved in Aspergillus fumigatus Internalization into Type II Human Pneumocyte A549 Cells. PloS one 6 (7):e21468. doi:10.1371/journal.pone.0021468
[28] Xu XY, Shi Y, Zhang PP, Zhang F, Shen YY, Su X, Zhao BL (2012) E-cadherin mediates adhesion and endocytosis of Aspergillus fumigatus blastospores in human epithelial cells. Chinese medical journal 125 (4):617-621
[29] Balloy V, Sallenave JM, Wu Y, Touqui L, Latge JP, Si-Tahar M, Chignard M (2008) Aspergillus fumigatus-induced interleukin-8 synthesis by respiratory epithelial cells is controlled by the phosphatidylinositol 3-kinase, p38 MAPK, and ERK1/2 pathways and not by the toll-like receptor-MyD88 pathway. The Journal of biological chemistry 283 (45):30513-30521. doi:10.1074/jbc.M803149200
[30] Yadav BS, Tripathi V, Kumar A, Khan MF, Barate A, Kumar A, Sharma B (2012) Molecular modeling and docking characterization of Dectin-1 (PAMP) receptor of Bubalus bubalis. Experimental and molecular pathology 92 (1):7-12. doi:10.1016/j.yexmp.2011.09.018
[31] Wolf AJ, Simmons RM, Goodridge HS, Underhill DM (2009) Polymorphisms associated with human Dectin-1 result in disrupted protein folding and loss of function (135.47). The Journal of Immunology 182 (1 Supplement):135.147-135.147
[32] Rosentul DC, Plantinga TS, Oosting M, Scott WK, Velez Edwards DR, Smith PB, Alexander BD, Yang JC, Laird GM, Joosten LA, van der Meer JW, Perfect JR, Kullberg BJ, Netea MG, Johnson MD (2011) Genetic variation in the dectin-1/CARD9 recognition pathway and susceptibility to candidemia. The Journal of infectious diseases 204 (7):1138-1145. doi:10.1093/infdis/jir458
[33] Fischer M, Spies-Weisshart B, Schrenk K, Gruhn B, Wittig S, Glaser A, Hochhaus A, Scholl S, Schnetzke U (2016) Polymorphisms of Dectin-1 and TLR2 Predispose to Invasive Fungal Disease in Patients with Acute Myeloid Leukemia. PloS one 11 (3):e0150632. doi:10.1371/journal.pone.0150632
[34] Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ (2008) Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cellular microbiology 10 (10):2058-2066. doi:10.1111/j.1462-5822.2008.01188.x
[35] Meyer-Wentrup F, Figdor CG, Ansems M, Brossart P, Wright MD, Adema GJ, van Spriel AB (2007) Dectin-1 interaction with tetraspanin CD37 inhibits IL-6 production. Journal of immunology (Baltimore, Md : 1950) 178 (1):154-162
[36] Tandale A, Joshi M, Sengupta D (2016) Structural insights and functional implications of inter-individual variability in beta2-adrenergic receptor. Scientific reports 6:24379. doi:10.1038/srep24379

LIST OF PUBLICATIONS

1. **Thakur R** and Shankar J (2017). Proteome profile of *Aspergillus terreus* conidia at germinating stage; Identification of probable virulent factors and enzymes from mycotoxin pathways. **Mycopathologia**, doi: 10.1007/s11046-017-0161-5. **(I.F 1.7; SCOPUS), UGC Journal list No. 37158**
2. **Thakur R** and Shankar J (2016). *In silico* Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens. **Front. Microbiol**. 7:192. doi: 10.3389/fmicb.2016.00192 **(I.F 4.1; SCOPUS)), UGC Journal list No. 17739**
3. **Thakur R**, Anand R, Tiwari S, Singh AP, Tiwary BN and Shankar J (2015). Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells? ***Front. Microbiol***.6:429. doi: 10.3389/fmicb.2015.00429 **(I.F 4.1; SCOPUS) , UGC Journal list No. 17739**
4. **Thakur R** and Shankar J. Strategies for gene expression in Prokaryotic and Eukaryotic system. In kalia, V.C, Saini, A.K, Saini, R.V, Sharma, D.K, Strategies for metabolic engineering in bioactive compounds and processes. **Springer** (In Press)

5. **Thakur R** and Shankar J. Proteome analysis revealed Jak / Stat signaling and cytoskeleton rearrangement proteins in human lung epithelial cells during interaction with *Aspergillus terreus*. **Current Signal Transduction Therapy (SCOPUS), UGC Journal list No. 14407**. (Under revision)

CONFERENCES/WORKSHOPS

1. **Thakur R** and Shankar J (2015) presented poster entitled "Heat shock proteins influence Aflatoxin biosynthesis in *Aspergillus flavus"* in National conference on 'Emerging Trends in Host-Microbe Interaction' organized by DAV University, Jalandhar, Punjab
2. **Thakur R** and Shankar J (2015) Attended National symposium on computational system biology organized by dept. of biotechnology and bioinformatics JUIT, March- 1^{8th}- 2^{0th} 2016, Waknaghat, Solan, H.P., India and NNMCB (Mohali Node), SERB and presented poster entitled "In silico analysis of single nucleotide polymorphisms in human Dectin-1 gene and their impact on fungal infections.
3. **Thakur R** and Shankar J (2015) Participated in conference on Recent Trends in Biomedical Engineering, Cancer Biology and Bioinformatics organized by "Krishi Sanskriti" at JNU, 28^{th} Nov., 2015, New Delhi and presented poster entitled "In-silico T-cell epitope mapping from antigens of *Aspergillus fumigatus* for potential candidate for *Aspergillus*-specific T cells"
4. 7^{th} Advances against Aspergillosis Manchester, United Kingdom, 3^{rd} -5^{th} March 2016. Identification and functional characterization of protein involved in germination of *Aspergillus flavus* conidia
5. Participated in a workshop on "innovation and intellectual property rights" organized by Jaypee University of information technology and H.P state department of Science and technology on 9^{th} December 2016.

ACKNOWLWDGEMENT

I want to thank Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, for providing facilities to carry out the work and Ph.D. fellowship.